ZOOLOGICAL SCIENCE **16**: 845–851 (1999)

Age Determination by Skeletochronology of the Japanese Salamander *Hynobius kimurae* (Amphibia, Urodela)

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ABSTRACT—We investigated lines of arrested growth (LAG) of long bone tissues in a total of 157 salamanders of *Hynobius kimurae* from Tokyo and Kyoto, Japan. The number of LAGs did not differ between femurs, humeri, and toe phalanges. We found that the first LAG is formed after the first overwintering. The number of LAGs varied from 5–14 ($\bar{x}$=8.8) in reproductive males and 7–12 ($\bar{x}$=9.4) in mature females in the Tokyo population, while in the Kyoto population, males and females had 6–20 ($\bar{x}$=9.1) and 7–17 ($\bar{x}$=9.9) LAGs, respectively. This suggests that the minimum maturation age in males is five yr in Tokyo and six yr in Kyoto, while females of both populations need at least seven yr. The female-larger sexual size dimorphism, recognized in each population of this species seems to be attributable to a greater growth rate in females after the age of male maturity. Body size growth was better in Kyoto than in Tokyo, with average adult SVL being 3.2–3.6 times and 2.2–2.3 times of SVL of metamorphs, respectively. Adults of the Kyoto population, like metamorphs, are smaller than those of the Tokyo population, and such a difference is considered to be derived not from the differential age at maturity or growth rate but from the size difference at metamorphosis. In both populations, clutch size does not correlate to female age, but older and hence larger females tended to lay larger eggs. This suggests that the female fitness increases with age, not through an increase in the number of offspring per clutch, but through an elevation of survivorship per offspring.

INTRODUCTION

In the biological studies of amphibians, accurate age determination of individuals has been one of the most important subjects. Historically, age estimation has long been attempted through the analyses of size frequency distributions of populations or by mark-recapture records of individuals. Recently, however, these classic techniques have been replaced or supplemented by more reliable methods of skeletochronology (Halliday and Verrell, 1988). Of the methods of age estimation using bone tissues, counting lines of arrested growth (LAGs) is most popular (Castanet and Smirina, 1990; Castanet *et al*., 1993).

Bone tissues of tailed amphibians are very simple in structure and LAGs are generally very clear and easily studied. Caetano and Castanet (1987) demonstrated the annual formation of one LAG in some species of the suborder Salamandroidea. Since then, quite a few studies have been made for members of this suborder including Ambystomatidae (Flageole and Leclair, 1992), Plethodontidae (e.g., Castanet *et al*., 1996), and Salamandridae (e.g., Caetano and Castanet, 1993; Caetano and Leclair, 1996). Results of these studies indicate that age determination is relatively easy in the salamandrids whose growth lines are evident, whereas some plethodontids have less clear lines that require special calculation procedures in determining their ages (e.g., Castanet *et al*., 1996).

In contrast to such a situation for Salamandroidea, which is regarded as the most advanced group among tailed amphibians, the study on the Siberian *Salamandrella keyserlingii* by Ledenzov (1986) has been the only attempt of the skeletochronological approach to members of Cryptobranchoidea, the most primitive urodelan group (Larson and Dimmick, 1993). Ecologically, the Cryptobranchoidea encompasses two groups of species with different breeding habits, i.e., lentic breeders (including *S. keyserlingii*) and lotic breeders (Sato, 1943). These groups also differ in the duration of larval period, presence or absence of larval overwintering, and the size at metamorphosis. Such differences predict differential growth patterns and population age compositions between the two groups. However, the scarcity of aging studies, especially on the lotic breeding members, has made it impossible to verify this ecologically and evolutionarily interesting predictions.

Hynobius kimurae is a lotic breeding salamander occurring in Honshu of Japan, and varies greatly in ecological traits among populations. Our previous study (Misawa and Matsui, 1997) revealed the presence of differences in the larval life history, adult body size, clutch size, and egg size between the

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Tokyo and Kyoto populations of this species. Based on such results, we suspected variations in metamorphic age and size as the major sources of adult body size variation between the two populations.

In this study, we studied LAGs in this species again using these two populations. We first investigated the timing of LAG formation and variation in the LAG number among different bones in *H. kimurae* to generalize the validity of age estimation using skeletochronology in the Hynobiidae, the major group of the Cryptobranchoidea. We then compared the growth pattern and the age at sexual maturity between the two populations on the basis of individual ages thus estimated so as to determine how differential metamorphic size affects the difference in the adult body size.

MATERIALS AND METHODS

We collected salamanders between 1988 and 1995 in small montane streams of Miyama-cho, Hachioji-shi, Tokyo (35°42'N, 139°13'E, alt. 450 m) and Kurama-Ninose-cho, Sakyo-ku, Kyoto (35°08'N, 135°45'E, alt. 550 m)(Misawa and Matsui, 1997). We sampled larvae between September and June, a period including the overwintering season (December–February) in Tokyo. We also collected young salamanders just after metamorphosis between September and November in Tokyo, and July and September in Kyoto. Adults were obtained from December to April in both localities when congregating in breeding streams for overwintering and subsequent breeding. As a result, we collected and examined a total of 157 specimens: six larvae, 24 young (10 metamorphs before, and 14 after, the first winter), 45 adult males, and 15 adult females from Tokyo; 22 young (all metamorphosed before the first winter), 30 adult males, and 15 adult females from Kyoto. Some of the males and all females used in this study were those used in Misawa and Matsui (1997).

We fixed salamanders in 10% formalin and later stored in 70% ethanol. For each specimen, we measured snout-vent length (SVL) from the tip of the snout to the anterior corner of the cloaca using a digital calliper to the nearest 0.05 mm. The data for adult females and their clutch size and egg diameter are those presented in Misawa and Matsui (1997).

We counted and measured growth arrested lines (LAGs) seen in the cross section of long bones (Castanet and Smirina, 1990). For preparing bone sections, we followed Kusano *et al.* (1995): After taking out femurs from preserved specimens, we washed the bones in the tap water for about one hr, decalcified in 5% nitric acid for about 30 min in young and 45–60 min in adults, and then washed in the running water for a night. Using a freezing microtome, we obtained sections (25 μm thick), stained them with Mayer's acid hemalum for 30 min in the room temperature, and then washed them for 60 min in the tap water.

We chose sections from the central regions of the diaphysis and counted the number of LAGs under a light microscope at 400×magnification. In order to compare the differences among different bones, we also examined sections of humeri and the third toe phalanges from five randomly selected adults.

At the same time, by using an ocular micrometer, we determined the average diameter of the innermost LAG by measuring its minimum and maximum diameters. For the sections of individuals that metamorphosed before the first winter, we obtained the average outer diameter of the bone in the same way, and compared it with the diameter of the innermost LAG of individuals that experienced winter seasons.

We analyzed the growth pattern in body size of salamanders by fitting the Logistic Model $L=a/(1+e^{c-bt})$ following Kubo and Yoshihara (1972). In this model, L is the SVL (mm) at time t (yr), and a is the asymptotic size; c is the constant determined by putting 0 to t; and b is the instantaneous growth rate.

For comparisons of yearly fluctuations in age composition of populations, a two-tailed Mann-Whitney's U test was performed to detect the presence or absence of differences in the frequency distributions. In order to examine relationships between age and reproductive parameters, we first performed analysis-of-covariance (ANCOVA). However, because there is a significant correlation between female body size and one reproductive parameter (egg diameter: Misawa and Matsui, 1997), and age might affect reproductive parameter through size, partial correlation analyses were then run, setting reproductive parameters as criterion, and age and SVL as explanatory variables. The significance level was set at 0.05.

RESULTS

LAGs in tissues of different bones

We could observe distinct LAGs in femurs of *H. kimurae* (Fig. 1). There was no breakage of LAGs by endosteal resorption, and counting of LAGs was very easy. In each of the five individuals for which LAGs in humerus, digit, and femur were compared with each other, the count did not differ among the bones. LAGs in humerus and digits were clear like in femurs and there was no endosteal resorptional damage, either.

LAG formation

The larvae from Tokyo had no LAG before the first winter (Fig. 1A), and this condition lasted during and after the overwintering season (from December to February). Similarly, young individuals that transformed before winter (collected from September to November) had no LAGs. The overwintered larvae showed the formation of one LAG first in June, about four months after the first winter (Fig. 1B). After that period, these larvae metamorphosed and had the complete first LAG before the second winter (Fig. 1C). The diameter of this first line ($\bar{x}\pm2SE=256.8\pm13.2$ μm) did not differ from the femur diameter in larvae or metamorphs collected before the first winter (273.9±10.0 μm; Students *t* test: $P>0.05$). From these results, it is obvious that the formation of the first LAG requires several months after the first inactive period, i.e., the first overwintering, regardless of the occurrence of metamorphosis. The line supposedly formed at metamorphosis (metamorphic line) was vaguely seen in only a few individuals. The first LAG was retained in adults from Tokyo (Fig. 1D), and its diameter (238.3±6.4 μm in males and 251.5±10.6 μm in females) was almost identical to the diameter of the first LAG in overwintered larvae and young, and also to the femur diameter of individuals collected before the first winter ($P>0.05$ in all comparisons).

In Kyoto, all larvae metamorphose before the first winter (Misawa and Matsui, 1997). Young individuals collected in autumn had no LAG in the femur as in those from the Tokyo population. Adults from Kyoto also retained the first LAG, and its diameter (174.2±6.4 μm in males and 170.2±9.8 μm) did not differ significantly from the femur diameter of just metamorphosed young (182.0±9.4 μm; $P>0.05$).

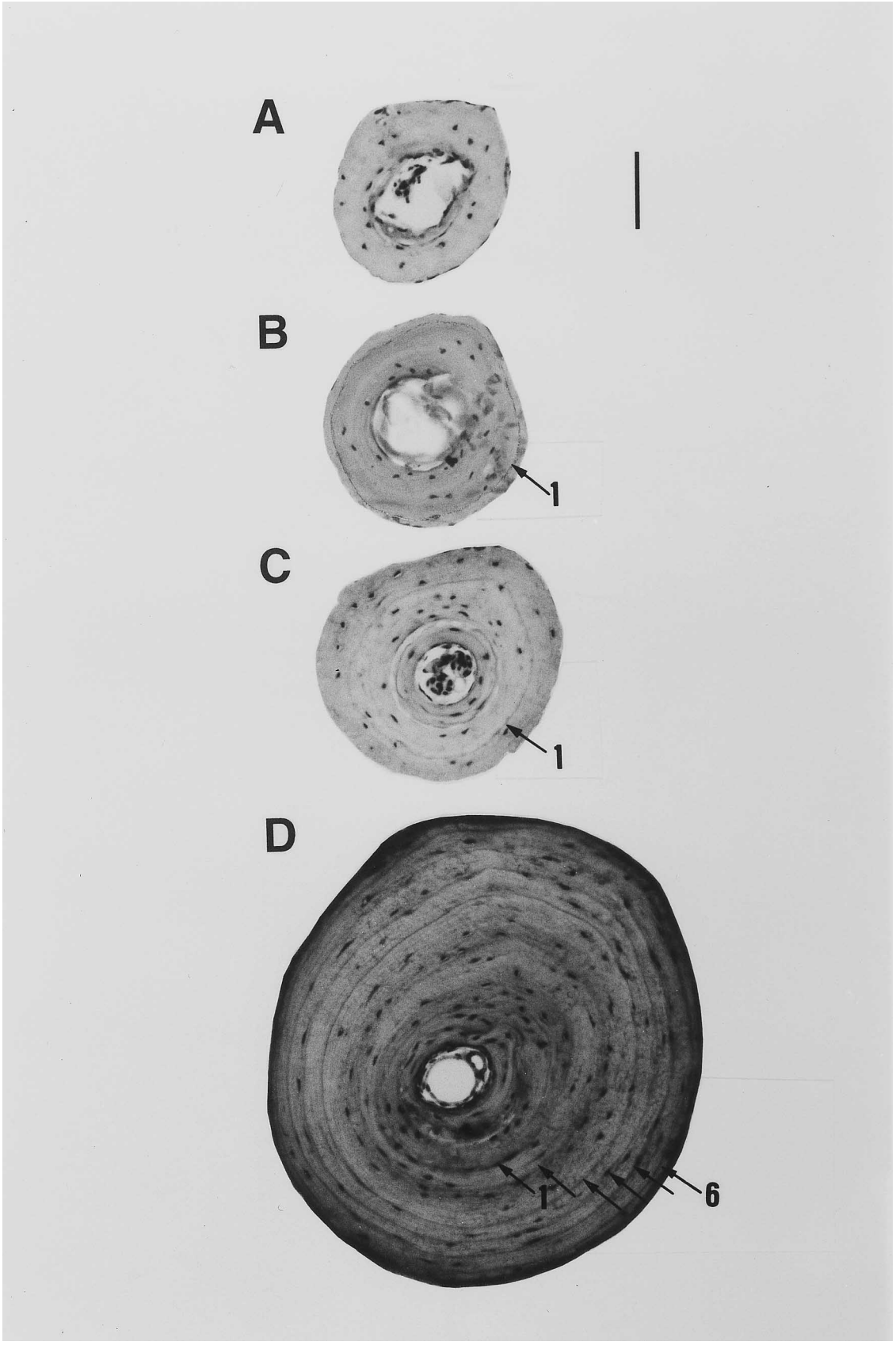

Fig. 1. LAGs seen in femurs of *H. kimurae* from Tokyo. A: a larva collected before the first winter (September); B: a larva collected shortly after the first winter (June); C: a young collected long after the first winter (September); D: an adult male of six yr old. Bar=100 μm.

Yearly fluctuation in age composition

As shown above, the innermost LAG is surely formed after the first overwintering, and is retained in adults. Consequently, the number of LAGs found in the bone tissues can be reasonably assumed as representing the individual age. Table 1 shows yearly fluctuations in age compositions of adult samples collected over a maximum of four breeding seasons (each from December to April). In males from Tokyo, the number of LAGs varied between 5 and 14, with the average (±2SE) of 8.8±0.7. Medians of LAGs varied yearly between 7–11. In females from Tokyo for which data are available for only three years, the range of the number of LAGs was 7–12 ($\bar{x}$±2SE=9.4±0.7), and yearly medians ranged from 9–10. In the comparison of age compositions between 1989–1990 and 1992–1993 breeding seasons in the Tokyo population, males were significantly older in the latter period than in the former (Mann-Whitney U test, $P<0.01$), whereas females showed no significant difference ($P>0.05$).

In adult samples collected from Kyoto in 1991–1992 and 1992–1993 breeding seasons, LAGs for males varied from six to 20 ($\bar{x}$±2SE=9.1±0.9) with their yearly median varying from 7.5–10.5, whereas those for females varied from eight to 17 ($\bar{x}$±2SE=9.9±1.2), with 9.5–10 yearly medians. Males collected in the 1992–1993 season were significantly older than in 1991–1992 season ($P<0.01$), but females did not differ in the two years ($P>0.05$).

Age at sexual maturity and longevity

In both the Tokyo and Kyoto populations, oviposition takes place between February and March and larvae hatch in April

Table 1. Yearly fluctuations in the number of LAGs in adults collected in breeding seasons (December–April).

Year	Males			Females		
	n	Range	(Median)	n	Range	(Median)
Tokyo						
1988–1989	6	5–10	(7.5)	1	10	
1989–1990	17	5–11	(7)	10	8–11	(9)
1992–1993	16	7–14	(11)	4	7–12	(9.5)
1994–1995	6	6–12	(9)	0	–	
Kyoto						
1991–1992	24	6–12	(7.5)	6	7–11	(9.5)
1992–1993	16	6–20	(10.5)	9	8–17	(10)

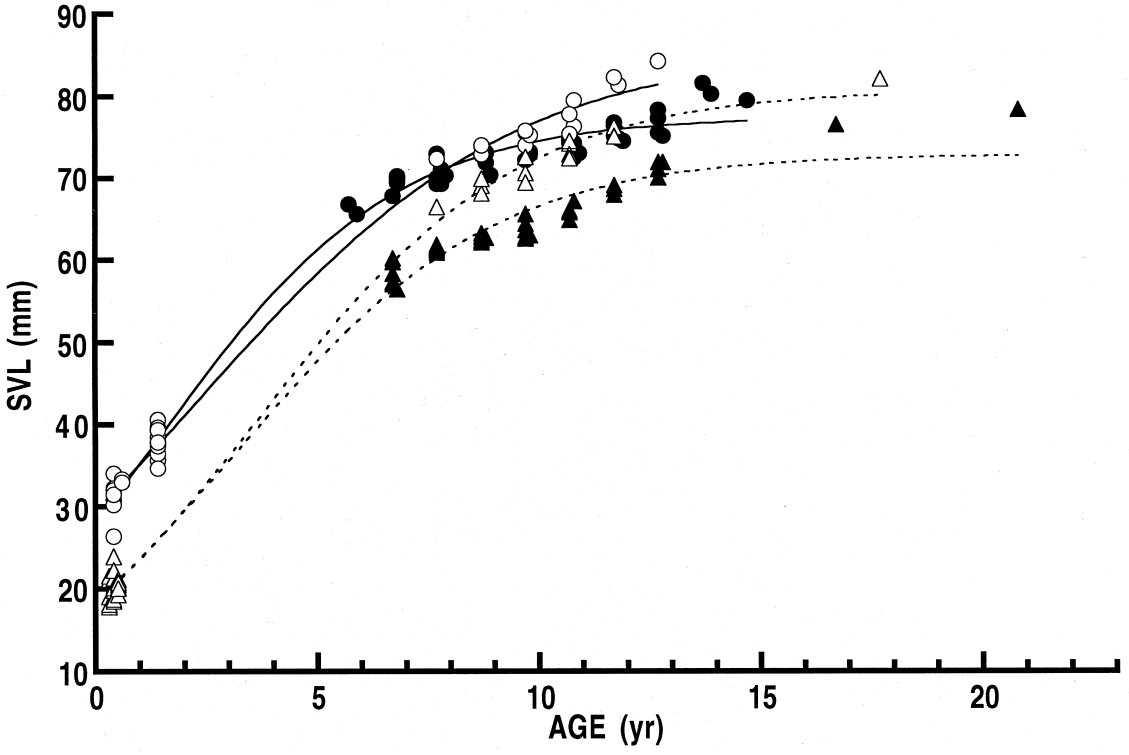

Fig. 2. Growth curves estimated by Logistic Model for males (filled symbols) and females (open symbols) of *H. kimurae* from Tokyo (circles) and Kyoto (triangles).

(Misawa and Matsui, 1997). Setting the start at the time of hatching, we estimated individual ages from the number of LAGs and dates of collection. In the Tokyo population, the minimum number of LAGs found in males that aggregated for breeding was five (Table 1), suggesting that it takes at least five yr for males to attain sexual maturity. On the other hand, breeding females had seven or more LAGs, and this suggests that they become sexually mature later than males. In the Kyoto population, minimum age at sexual maturity is estimated to be over six yr in males and seven yr in females. Sexual maturity in males is thus supposed to occur one year earlier in Tokyo than in Kyoto, whereas it occurs simultaneously in females of the two populations.

The age of the oldest individuals, over 14 yr (male) in Tokyo and 20 yr (male) in Kyoto (Table 1), suggests that the longevity of this species is fairly long, usually over 10 years.

Growth

The growth curves estimated by Logistic Model are shown in Fig. 2. In the Tokyo population, males had a larger instantaneous growth rate (b±2SE=0.3766±0.0288) than females (b±2SE=0.2783±0.0462), and after sexual maturity, growth of males slowed down more quickly than in females. Average adult SVL was 2.2 times in males and 2.3 times in females of the SVL at metamorphosis. Sexes did not differ in the instantaneous growth rate in the Kyoto population (b±2SE= 0.3418±0.0222 in males and 0.3371±0.0258 in females), but the decrease of growth rate in females was smaller than in males even after sexual maturity. Compared with the metamorphs, adults were larger by 3.2 times in males and by 3.6 times in females. Thus, in both sexes, size of adults in relation to metamorphs was much larger in the population from Kyoto than that from Tokyo, although the size of metamorphs is much smaller in the former population.

Female age, ovarian egg number, and egg diameter

In both populations, ovarian egg number did not correlate to the female age ($P>0.05$). By contrast, a positive correlation ($P<0.05$) was found between female age and egg diameter, indicating that older females tend to lay larger eggs in both populations. However, when statistically "pure" action of age (X_2) on egg diameter (X_1) was estimated by partial correlation analyses using female SVL as another explanatory variable (X_3), age had no significant correlation to egg diameter for either population when SVL was held constant ($r_{12.3}$= 0.037, dF=12, $P>0.05$ for Tokyo and $r_{12.3}$=0.105, dF=12, $P>0.05$ for Kyoto).

DISCUSSION

Of the urodelan species examined skeletochronologically by now, LAGs in the Salamandridae (Francillon, 1979; Caetano *et al*., 1985; Verrel and Francillon, 1986; Montori, 1990; Forester and Lykens, 1991), Ambystomatidae (Flageole and Leclair, 1992), and Proteidae (Senning, 1940) are reported to be generally evident and directly applicable to age determination. On the other hand, age determination proved to be not easy in some species of the Plethodontidae because their LAGs are not always clear (Houck and Francillon-Vieillot, 1988; Wake and Castanet, 1995). Some authors attributed the variation in the clarity of LAGs seen among different species to the difference in the histological properties of the bone, regulated by genetic factors (e.g., Castanet *et al*., 1996). In a hynobiid, *Salamandrella keyserlingii*, LAGs have been reported to be generally clear (Ledenzov, 1986), like in our *H. kimurae*. From these findings, we assume that hynobiids are similar to species of the Salamandroidea other than Plethodontidae in the histological properties of growth lines that are related to the degree of formation in bony material, type of the bone, and quality of the LAG.

It has been demonstrated experimentally in *Triturus marmoratus* that the increase of the LAG number corresponds to annual periodicity of life history (Caetano and Castanet, 1987), and this finding forms the basis for using LAGs in the salamandrid age determination. From the present comparisons of larvae before and after overwintering, we could confirm the formation of a LAG not before, but after the first and presumably all successive winters in *H. kimurae*. From this result, it is almost certain that the formation of LAG occurs annually, closely relating to inactive, winter seasons, and that we can estimate ages of *H. kimurae* by the present technique with a high confidence like in salamandrid species. Unlike some plethodontid species that contain many individuals with the first LAG damaged through the endosteal resorption, and require a back calculation by using individuals retaining the first LAG (Castanet *et al*., 1996), all individuals of *H. kimurae* retained the first LAG, and could be aged without special calculation. On the other hand, some populations of *Salamandrella keyserlingii* are reported to include individuals with partially resorbed first line (Ledenzov, 1986). Further study is required to evaluate the degree of the occurrence of resorption among hynobiid salamanders. Because the first LAG is formed after the first winter both in larvae and young but the metamorphic line remains incompletely in few individuals in *H. kimurae*, we cannot determine skeletochronologically whether or not an individual had already metamorphosed before the first winter. This also offers a problem to future studies.

In studies of skeletochronology in urodeles, femurs (Forester and Lykens, 1991), humeri (Caetano and Leclair, 1996), digits (Ledenzov, 1986), and combination of femurs or humeri and digits (Francillon-Vieillot *et al*., 1990; Caetano and Castanet, 1993) have been examined. In *T. marmoratus*, Caetano and Castanet (1993) reported that digital bones can be used for age estimation, although intervals between each LAG in these bones are narrower and more irregular, and hence less easily counted, than in humeri. By contrast, counting LAGs in digits of *T. vulgaris* has been reported to be difficult because tissues include much cartilaginous components that are easily resorbed (Verrel and Francillon, 1986). *Hynobius kimurae* investigated here showed identical numbers of LAGs in all the three bones, and the digital LAGs were

clear and easily counted. Because age estimation from digits has also been successfully done in *Salamandrella* (Ledenzov, 1986), ages of hynobiids in general are supposed to be known only from phalanges. Using femurs or humeri gives animals much more damage, or in the worst case, will eventually cause their death (Halliday and Verrell, 1988), from which we must refrain, as long as possible, on the viewpoint of species conservation. Toe clipping has been the most popular technique for individual marking, and would give minimum damage on the survival of animals. For the estimation of ages of salamanders in nature, utilization of clipped digits is thus strongly recommended.

In both populations from Tokyo and Kyoto, adult *H. kimurae* shows clear sexual size dimorphism (SSD) in SVL in which females are larger than males (Misawa and Matsui, 1997). Such SSD in urodeles is considered due either to the larger growth rate in females (Caetano and Leclair, 1996) or to the decrease of growth rate only in males after their maturation (Houck, 1982; Caetano and Castanet, 1993). *Hynobius kimurae* seems to fall in the second case. In both populations, growth rate of males, originally similar to that of females, quickly lowered at six yr when they begin to participate in reproduction. By contrast, females continued growth and sexually mature. Further, the subsequent decrease of growth rate in females is smaller than in males, making SSD larger in older ages. Such a difference in growth rate between sexes after sexual maturity is regarded as the main source of SSD in this species. On the other hand, sexually similar growth rate before maturation seems to force females to take longer time for maturation than males.

Intraspecific variation in body size of urodeles is considered to be related to differences in individual longevity, age at first reproduction, and growth rate, and these differences are reported to be caused by latitudinal and altitudinal differences between populations (Caetano and Castanet, 1993) or by differential survival rate and reproductive success of individuals (Caetano and Leclair, 1996). Of the two populations of *H. kimurae* studied here, the Tokyo population is larger in adult body size (as represented by SVL) than the Kyoto population in both sexes. There is no marked difference in latitude or elevation of their localities, but the characteristics of the larval habitats drastically differ between these two populations (Misawa and Matsui, 1997). In Kyoto, the stream dries up in fall and all larvae metamorphose before winter. By contrast, the stream does not dry up in Tokyo and only few of the larvae metamorphose before winter, leaving most others to transform in the next year. Moreover, the size at metamorphosis is much larger in the Tokyo population even if metamorphosis takes place before winter (Misawa and Matsui, 1997). The growth curves of the two populations indicate that the marked difference in the adult body size is not attributable to differences in the age at sexual maturity or growth rate, and we, at present, suspect that this is most strongly affected by the differential metamorphic size which is derived from differential larval life histories in the two populations. Accumulations of data for survival rate and reproductive success in future studies are strongly desired to explore other possibilities and verify this ad hoc hypothesis.

The genus *Hynobius* includes many species that breed either in lotic or lentic waters, but no species have been studied for their age characteristics, except for lentic breeding *H. tokyoensis* (Kusano, 1982). From the growth rate of metamorphosed young in captivity, this author estimated the age at first reproduction in females of this species to be five yr. This value is much smaller than the age at first reproduction in females of lotic breeding *H. kimurae* (7–8 yr), and this difference, if correct despite the methodological difference in age estimations for these species, may be related to their different breeding habits. In contrast to lentic breeding salamanders that lay a large number of small eggs, lotic breeders including *H. kimurae* lay a small number of large eggs. Our results showed that older females of *H. kimurae* tend to lay larger eggs through larger body size. The reason for this tendency would be that salamanders of older ages, and hence approaching asymptotic size, have lower growth rates and can utilize surplus energy for higher fitness through greater reproductive effort. In doing so, they use this energy not to increase the clutch size, but to increase the size of each egg. This strategy may be common with the property of the lotic breeding salamanders in general, for which selection would favor greater ovum, and hence hatchling, size. Additional studies by skeletochronology would prove to be very effective to test this hypothesis from a demographic viewpoint.

ACKNOWLEDGMENTS

We thank T. Kusano for teaching techniques of skeletochronology, and H. Ota for critical comments on the manuscript. We also thank an anonymous reviewer for valuable comments.

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(Received May 7, 1999 / Accepted June 7, 1999)